Free Radical Activity of PM$_{10}$: Iron-mediated Generation of Hydroxyl Radicals

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The purpose of this study was to test the hypothesis that particulate matter ≤ 10 μm in aerodynamic diameter (PM$_{10}$) particles have the ability to generate free radical activity at their surface. We collected PM$_{10}$ filters from the Edinburgh, United Kingdom, Enhanced Urban Network sampling site, removed particles from the filter, and tested their ability to cause free radical damage to supercoiled plasmid DNA. We found that the PM$_{10}$ particles did cause damage to the DNA that was mediated by hydroxyl radicals, as shown by inhibition of the injury with mannitol. The PM$_{10}$-associated hydroxyl radical activity was confirmed using a high-performance liquid chromatography-based assay to measure the hydroxyl radical adduct of salicylic acid. Desferrioxamine abolished the hydroxyl radical-mediated injury, which suggests that iron was involved. Analysis of PM$_{10}$ filters confirmed the presence of large amounts of iron and leaching studies confirmed that the PM$_{10}$ samples could release substantial amounts of Fe(III) and lesser amounts of Fe(II). To investigate the size of the particles involved in the hydroxyl radical injury, we centrifuged the suspension of PM$_{10}$ to clarity, tested the clear supernatant, and found that it had all of the suspension activity. We conclude, therefore, that the free radical activity is derived either from a fraction that is not centrifugeable on a bench centrifuge, or that the radical generating system is released into solution. — *Environ Health Perspect* 105(Suppl 5):1285–1289 (1997)

Key words: PM$_{10}$, air pollution, free radical, oxidative stress, environmental lung disease

Introduction

Recent research has confirmed the adverse health impacts of particulate air pollution in causing acute mortality, impairment of lung function, and exacerbation of airway diseases such as chronic obstructive pulmonary disease (COPD) and asthma (1,2). PM$_{10}$ is the commonly used measure of respirable environmental suspended particulate material (SPM) and represents airborne particles less than 10 μm aerodynamic diameter collected with 50% efficiency; smaller particles are also collected with variable efficiency (3). In the United Kingdom, a standard of 50 μg/m^3 PM$_{10}$ was recently recommended by a Department of the Environment committee (3). The average PM$_{10}$ concentration for British cities ranges between 10 and 50 μg/m^3, with short-term peaks extending to 100 μg/m^3 (3,4), but in other countries much higher levels can be experienced.

Little data are available on the detailed composition of PM$_{10}$ and the composition is highly variable between locations (3,4). The association between mortality and pulmonary symptoms with SPM in diverse geographic locations such as New York, London, Utah, and Philadelphia, Pennsylvania, where the sources ranged from predominantly coal combustion to automobile emissions to a steel mill (5), suggests that the detailed composition of the SPM is not critical but that the total mass of fine particles is important. PM$_{10}$ particles can be in very fine size ranges (6). Particles in the 20 nm size range are common, and represent relatively small masses but large particle numbers (7).

It has been hypothesized that direct interactions between deposited fine particles and the pulmonary epithelium in the terminal bronchiolar/proximal alveolar regions is critical for harmful effects of PM$_{10}$ particles (8). We extended the hypothesis to suggest that PM$_{10}$, particularly an ultrafine component of it, together with other ultrafine materials, may have free radical activity that contributes to its toxicity. In this paper we suggest that, as in findings with other particles, free radicals may arise from Fenton chemistry involving iron present on the particles (9–11). Additionally, we investigated whether iron could be leached from PM$_{10}$ particles to a greater degree at the acid pH found in the phagolysosome (12).

Materials and Methods

Preparation of PM$_{10}$

The PM$_{10}$ was provided on filters taken directly from the tapered element oscillating microbalance in U.K. Enhanced Urban Network monitoring site stationed in Princes Street Gardens in Edinburgh. The filters were supplied by J. Hunter, Edinburgh District Council, Department of Environmental Services. Filters were quartered, and each quarter was placed in 100 μl sterile H$_2$O, 4 mM mannitol (BDH Chemicals, Poole, U.K.) or 4mM desferrioxamine-B (DSF-B) (Sigma Chemicals, Poole, U.K.) solution as required. Each sample was sonicated in a sonicating water bath for 30 sec and vortexed vigorously to remove PM$_{10}$ from the filter into solution. Blank filters without PM$_{10}$ were similarly treated for control purposes. The mean ± SEM weight of particulate matter on the filters was 996.2 ± 181.8 μg/filter. Although it is impossible to quantify the weight of particulates removed by sonication, we estimated by comparative turbidometry against carbon black standards that with our standard procedures, 10 to 30% of the particulate matter was removed from the filters into solution. In some

experiments, PM_{10} samples prepared in either H_2O or DSF-B were centrifuged for 10 min at 13,000 rpm to remove large insoluble particles from the solution. The resulting clear supernatant was added to the DNA assay at the same concentrations as the uncentrifuged PM_{10} solutions.

Iron Release from Particles

Iron release was measured under the two pH conditions of pH 4.6 and 7.2. PM_{10} samples were suspended in either pH 4.6 or 7.2 citric acid/$NaHPO_2$ (BDH Chemicals) buffer as described above. Following an 8-hr incubation at 37°C in a rotating incubator, larger visible particles were removed by centrifugation at 2500 rpm for 10 min. By our estimation, each quarter filter should provide a maximum of 75 µg PM_{10} particles. Iron concentration released after 8 hr was determined by the addition of either 4 mM DSF-B (Sigma Chemicals) for FeIII determination, or 4 mM ferrozine (Sigma Chemicals) for Fe(II) determination. Quantification of the particle-leached Fe(II) or Fe(III) was measured by spectrophotometry at 562 and 430 nm, respectively, and comparison with Fe(II) or Fe(III) standards of $FeSO_4$ and $FeCl_3$ (10). The limit of sensitivity of this assay was 0.5 nmol. The amount of iron leached from 74.7 µg of particles after an 8-hr incubation was expressed as nanomol Fe(II) or Fe(III).

Free Radical Activity of PM_{10}

Plasmid Assay. Two hundred and ninety nanograms $\phi X174$ RF1 DNA (Gibco Europe, Paisley, U.K.) was incubated with PM_{10} particles or PM_{10} particles treated as described above (10). Briefly, all DNA control and treatment samples were incubated for 8 hr at 37°C prior to agarose gel electrophoretic separation of the supercoiled, relaxed coil, and linear fragments of the DNA. The amount of supercoiled DNA present after treatment, compared to control, indicated the level of free radical-mediated damage to the plasmid. From our estimation of particles removed from each filter, the weight of particles in 5 µl should be 3.7 µg/assay. A typical agarose gel photo of these treatments is seen in Figure 1.
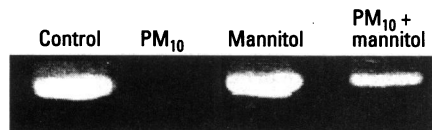
Figure 1. Typical result from a plasmid DNA injury assay. Note depletion of DNA by PM_{10} and partial protection with mannitol.

Mannitol was added to inhibit hydroxyl radical, at a concentration chosen from dose–response studies as the lowest concentration that caused maximal inhibition.

High-performance Liquid Chromatography Assay. This assay is based on the ability of particle-derived hydroxyl radicals to form the derivative 2,3 dihydroxybenzoic acid (2,3 DHBA) from salicylic acid. One quarter PM_{10} filter was sonicated in 250 µl of 25 mM salicylic acid in citrate/acetate buffer pH 3.85. The filter was then removed and the salicylic acid/PM_{10} suspension incubated for 16 hr at 37°C. The suspension was centrifuged to clarity and filtered through a 0.22-µm filter. One hundred microliters of this supernatant was loaded onto a 5 mm UltroSphere octadecyl silane 250 mm high-performance liquid chromatography (HPLC) column (Alltech, Carnforth, Lancashire, U.K.) and eluted with citrate/acetate buffer. Elution from the column was monitored using an ultraviolet detector, which automatically recorded the elution time of peaks. In control experiments, reagent 2,3 DHBA eluted at 7 min, compared to 15 min for salicylic acid. Using standard concentrations of 2,3 DHBA, we calculated the amount of conversion of salicylic acid to 2,3 DHBA caused by PM_{10}. A blank PM_{10} filter had no effect in this assay.

Scanning Electron Microscopic Energy Dispersive X-ray Analyses and X-ray Fluorescence Analysis

A PM_{10} sample was examined on the filter in a Cambridge Stereoscan S90 scanning electron microscope (ISS, Manchester, U.K.), and qualitative analysis carried out via the attached Link energy dispersive X-ray analyzer (EDXA) (Link Analytical Ltd, High Wycombe, U.K.). By focusing the electron beam on the fibers of the filter, we obtained an elemental analysis on the filter matrix material. Focusing on the clumps of particle visible between the filter fibers allowed analysis of the elements in the PM_{10} particles themselves. The EDXA spectra showed elements from atomic number 11 (sodium) upwards.

X-ray fluorescence analysis was carried out on an untreated PM_{10} filter using the Rigaku Model 2000 analyzer (Rigaku, Japan), which exposed the entire central 10 mm of the filter to the incident X-ray beam (generated on a rhodium target). The resulting element fluorescence emission from the sample was passed through collimators and diffracted by a lithium fluoride crystal to fall on a scintillation counter,

which scanned through the 2-ϕ range 0 to 99°. Only elements with an atomic number of 9 (fluorine) or greater were recorded.

Statistical Analysis

We used the Minitab statistical analysis program (Minitab, State College, PA) and 1- or 2-way analysis of variance to determine the significance of treatment effects. The Tukey multiple comparison function was used when required.

Results

Free Radical Activity of PM_{10}

Typical output from the DNA plasmid assay is shown in Figure 1, where there is depletion of the supercoiled DNA band by PM_{10}, and protection of the effect by mannitol. When this was quantified by scanning laser densitometry the results were as shown in Figure 2.

In HPLC eluate, a 7-min peak was found in PM_{10}-treated salicylic acid (Figure 3). The standard curve represented 2.1 nmol 2,3 DHBA, which, assuming 1:1 stoichiometry with salicylic acid, means that 2.1 nmol of hydroxyl radical is generated by a quarter filter of PM_{10}.

Effect of Desferrioxamine and Centrifugation on the Free Radical Activity of PM_{10}

When the iron chelator desferrioxamine was added to the plasmid assay, free radical activity was dramatically ameliorated, as shown in Figure 4. Further, when the PM_{10} was centrifuged to clarity and the clear supernatant tested, plasmid depletion in amounts similar to those of the whole PM_{10} suspension was noted (in Figure 4). This was again inhibitable by desferrioxamine.
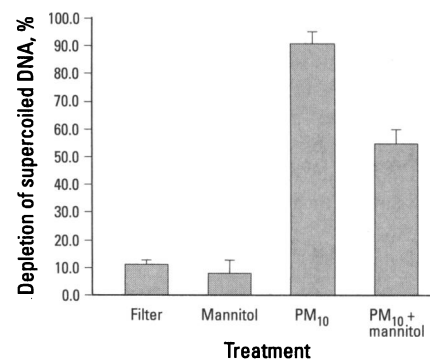
Figure 2. Quantified depletion of supercoiled DNA by blank filter, mannitol, PM_{10}, and PM_{10}+mannitol. Data are shown as mean+SEM and there was a significant protection of the PM_{10} effect with mannitol ($p<0.05$).
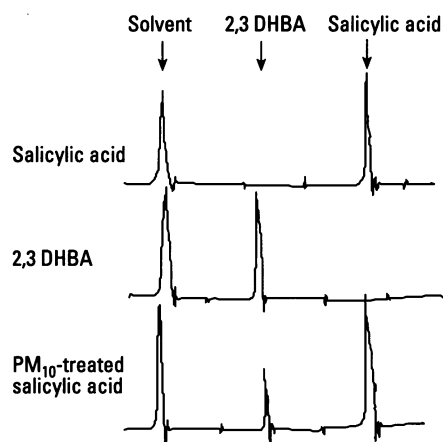
Figure 3. Traces from HPLC elution profiles of untreated salicylic acid, 2,3 DHBA standard, and PM₁₀-treated salicylic acid. Note the appearance of a 2,3 DHBA peak on treatment of salicylic acid with PM₁₀.
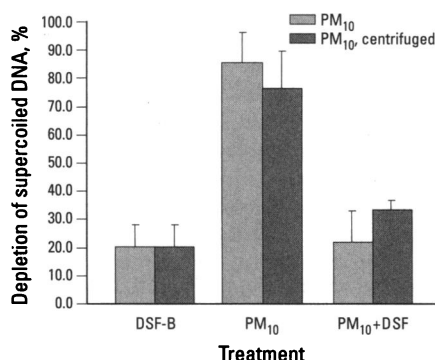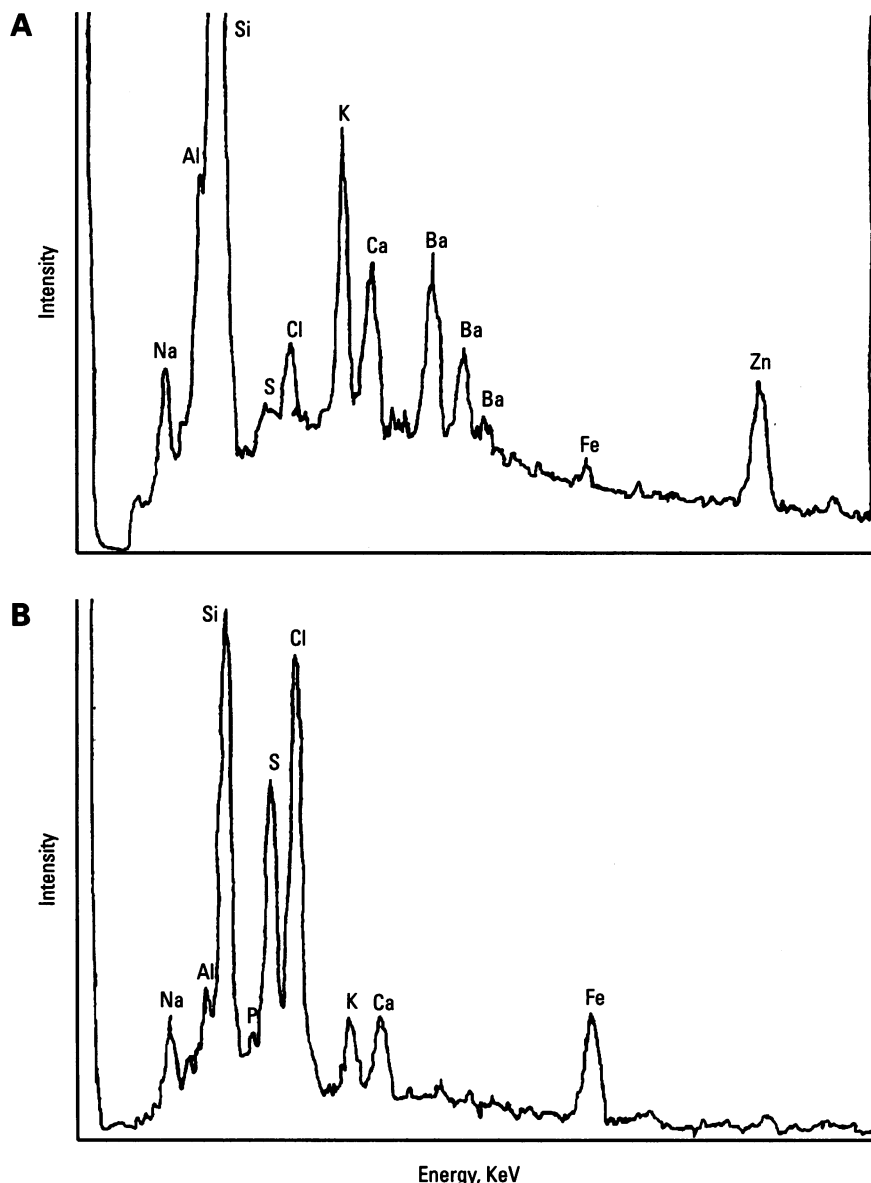

Figure 4. Effects of PM₁₀ and clear supernatant from centrifuged PM₁₀ on plasmid DNA; effect of DSF-B on the DNA depletion. Significant effect of DSF-B compared to PM₁₀ ($p<0.05$). Data are shown as mean + SEM.

Figure 5. Elemental analysis scan of a PM₁₀ filter. (A) The signal when the beam is focused on the filter fibers; (B) the scan obtained when the beam is focused on particles on the filter.

Iron in PM₁₀ Samples

Iron was measured directly on PM₁₀ filters by scanning electron microscopy with an elemental analysis facility, by X-ray fluorescence, and in leachates from PM₁₀, which were obtained using buffer at two pHs.

Scanning electron microscopy of the filter revealed that it was composed of fibers with a distinctive elemental profile characterized by Zn, Ba, Fe, K, Al, and S peaks (Figure 5A). When the beam was focused on particles (avoiding the filter fibers), a very different elemental profile was obtained: no Ba or Zn, diminished K and Ca peaks compared to the filter fibers, and substantial Fe, S, and Cl peaks (Figure 5B). X-ray fluorescence analysis yielded the proportions of major elements: Cl, 21.9%; Fe, 18%; Si, 14.9%, and Na, 10.5%.

Figure 6 shows the release of Fe(II) and Fe(III) from PM₁₀ at pH 4.5 and 7.2, respectively, which confirms that large amounts of Fe(III) and small amounts of Fe(II) are released, and that there was more release at acid pH.

Discussion

We recently advanced a unifying hypothesis to explain the pulmonary and extrapulmonary effects of PM₁₀ particles, which was centered around their proposed ability to penetrate the pulmonary interstitium and cause interstitial inflammation (8).

This hypothesis evolved from the original studies of Ferin et al. (13), which demonstrated that ultrafine titanium dioxide was highly inflammogenic and rapidly crossed the epithelium to initiate interstitial inflammation. We demonstrated that ultrafine TiO₂ has free radical activity (14) and hypothesized that this is a factor that enhances interstitial transfer of particles. The relatively huge surface area/unit mass of ultrafine material (7) could be an important factor that leads to oxidative stress to the epithelium and to macrophages. We demonstrated in in vivo and in vitro
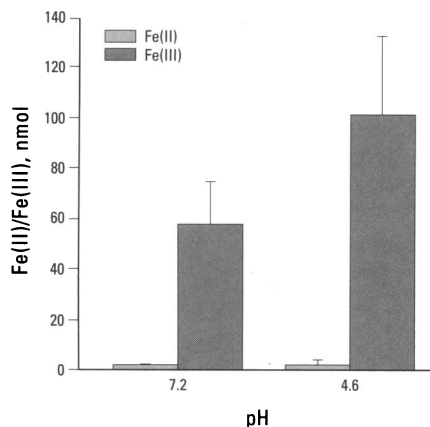
Figure 6. Iron leaching from PM_{10} at pH 4.5 and 7.2. Data are shown as mean + SEM.

models that oxidative stress leads to loss of epithelial integrity (15,16), which could favor interstitial transfer of particles.

We demonstrate here that PM_{10} particles have free radical activity as shown by their ability to deplete supercoiled plasmid DNA via $OH^{\bullet}$ injury, as confirmed by the protection afforded by mannitol against the PM_{10} injury. Experiments using desferrioxamine and centrifugation revealed that the $OH^{\bullet}$ are generated through reactions that involve Fe, and that this resides either in a soluble or ultrafine fraction.

PM_{10} particles are thus similar to other pathogenic particles with free radical activity: asbestos (10), glass fibers (10), coal (17), and quartz (18). Iron from asbestos (19,20) and asbestos bodies (21) also has a role in the generation of free radicals. The potentially huge surface area for surface-complexed iron (20), or release of soluble iron presented by an ultrafine fraction of PM_{10}, may cause them to deliver a severe

oxidative stress to cells or lung lining fluid that they encounter.

Not all of the DNA scission was mannitol inhibitable, which demonstrated that other mechanisms for DNA scission are present—not surprising in a heterogeneous mixture such as PM_{10}. PM_{10} samples released substantial amounts of iron, principally Fe(III), but also trace amounts of Fe(II), which confirms that iron could be released in lung lining fluid and in increased amounts in the macrophage phagolysosome. We assume that there was a reducing source in the PM_{10} that led to redox cycling with production of Fe(II), which would enhance the capacity to generate $OH^{\bullet}$. The identity of the reducing component or components is unknown.

X-ray fluorescence and analytical scanning electron microscopy revealed a large proportion of Fe in the PM_{10} sample. The Fe proportion confirmed previous reports that iron, as various salts, is present in SPM samples (22) to the calculated concentration of 0.5 $\mu g/m^3$ of air (23).

The present data may be of value to explain the association between high PM_{10} levels and adverse health effects. For instance, the ability of asbestos to stimulate macrophages to release tumor necrosis factor-alpha can be abolished by chelation of fiber iron (24), which highlights the importance of oxidative stress in particle-mediated cytokine gene transcription in macrophages (25). Asbestos-stimulated transcription of cellular oncogenes c-fos and c-jun is inhibited by the thiol antioxidant N-acetyl-L-cysteine (26) in mesothelial cells. PM_{10} particles, via the free radical activity demonstrated here, may alter the redox balance of key lung cells and lead to the transcription of proinflammatory cytokines.

The data here contribute a plausible hypothesis for the adverse health effects of PM_{10} that needs to be further tested. If the iron is present in substantial quantities on the ultrafine fraction, then the relatively huge surface area presented by this fraction could lead to severe oxidative stress in the lung lining fluid and at contact between particles and cells. Our data showed increased leaching of iron at acid pH and suggests that, in the macrophage phagolysosome, low pH could favor release of iron and provide the opportunity for enhanced generation of free radicals. The possibility that PM_{10} causes exacerbation of airway diseases by an oxidative mechanism, as suggested here, is particularly plausible in view of the authors' own evidence that patients with airway diseases have a measurable deficit in their antioxidant defenses (27). We demonstrated that smokers and patients with exacerbations of COPD and asthma have a systemic lowering of their antioxidant defenses, as measured by a test that detects total antioxidant activity in plasma (27). The deficit in antioxidant defenses shown in plasma is likely to originate from the lungs, in view of the known oxidative stress resulting from the activities of inflammatory leukocytes. These patients are therefore likely to have oxidative stress in the epithelium and lung lining fluid. Further exposure of the oxidatively stressed lung to PM_{10} particles with free radical activity would represent significant additive oxidative stress that could lead to an aggravation of their condition. The resulting interstitial transfer of particles and pulmonary inflammation could be a significant factor in promoting cardiovascular mortality (8).

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